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(54) Title: SCREEN FOR AXON VIABILITY

(57) Abstract: A method for determining the viability of an axon comprises: (i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC); (ii) determining whether sGC is stimulated in the axon; and (iii) determining thereby whether the axon is viable.

areas (Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997). A major mechanism for NO signal transduction is activation of the enzyme soluble guanylyl cyclase (sGC), which causes the formation of cGMP from guanosine 5'-triphosphate (GTP). This pathway appears to mediate many of the physiological actions of NO in the CNS and elsewhere (Ignarro, 1991; Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997; Hobbs, 1997).

Summary of the invention

We have unexpectedly found that the rat optic nerve, a CNS white matter tract which lacks synapses and is composed mainly of glial cells and axons, is capable of generating large quantities of cGMP in response to NO and that this response is confined to the axons. This discrete localization, together with the fact that cGMP formation requires high energy phosphates that are lacking in non-viable tissue, indicated that the response can serve as a sensitive marker for optic nerve axon viability.

The finding that NO leads to cGMP formation in optic nerve cell axons is surprising. Previous evidence has indicated that, in the CNS, the NO-cGMP signalling pathway is primarily associated with synapses, yet synapses are absent from the optic nerve. Also, the neurones giving rise to the optic nerve axons, the retinal ganglion cells, do not appear to react to NO in the same way. In bovine or rat retinae, little or no cGMP immunostaining was observed in these cells in response to NO-donor compounds.

According to the present invention there is thus provided a method for determining the viability of an axon comprising:

(i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);

(ii) determining whether sGC is stimulated in the axon; and

(iii) determining thereby whether the axon is viable.

The invention also provides:

- a method for identifying a substance capable of protecting an axon from loss

accumulation in isolated adult rat optic nerves. (b) Protection of the cGMP response to 100 μM DEA/NO of OGD-treated optic nerves (shaded columns) by removal of Ca^{2+} ($0Ca^{2+}$) or Na^{+} ($0Na^{+}$) or addition of TTX (1 μM). All 3 treatments significantly restored cGMP level (P <0.001). Data are means ± S.E.M (n = 4-9).

Figure 2 shows protection against OGD-induced loss of optic nerve cGMP response to 100 μM DEA/NO by lamotrigine and analogues. Nerves kept in aCSF throughout are indicated by the open columns; nerves subjected to OGD are shown in shaded columns; *P < 0.02; **P < 0.0001 versus OGD alone (n = 4-12).

Figure 3 shows the histology and cGMP immunohistochemistry in control and OGD-treated optic nerves. (a) Semithin longitudinal section of untreated optic nerve following 5 h incubation. (b,c) cGMP immunostaining in longitudinal frozen sections of nerves incubated without (b) or with (c) DEA/NO for 5 min. (d-f) Semithin sections showing control histology in a transversely-cut optic nerve (d) and cGMP immunostaining in transverse (e) and longitudinal (f) sections of DEA/NO-treated nerves. (g-i) Semithin cross-sections showing the histology of optic nerves subjected to 1 h of OGD in the absence (g) and presence of BW619C89 (100 μM, h), or 1 μM TTX (i) followed, in each case, by 90 min recovery in normal aCSF. (j-l) cGMP immunohistochemistry of longitudinal frozen sections from DEA/NO-stimulated nerves previously subjected to 1 h OGD in the absence (j) or presence of BW619C89 (100μM, k) or TTX (1 μM, l). The DEA/NO concentration was 100 μM in all cases. Key: short arrows, axons; large arrowhead, oligodendrocyte; double small arrowheads, astrocyte soma; open arrows, band of glial cells; curved arrow, astrocyte processes. Scale bar (10 μm shown in a) applies to all micrographs.

Detailed description of the invention

The present invention provides a method for determining the viability of an axon which consists essentially of the following steps:

(i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);

activators of sGC include nitric oxide (NO), 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), carbon monoxide (CO) or YC-1 and CO. A combination of YC-1 and CO is a very effective activator of sGC.

Stimulators of sGC may be supplied in any way. For example, NO may be supplied in the form of an NO donor. This is particularly suitable if the assay is carried out in an aqueous environment. Suitable NO donors include organic nitrates (eg. glyceryl trinitrate), nitrites (eg. amyl nitrite), inorganic nitroso compounds (eg. sodium nitroprusside), sydnonimines (eg. molsidomine, 3-morpholinosydnonimine), S-nitrosothiols (eg. S-nitroso-L-cysteine, S-nitrosoglutathione, S-nitroso-N-acetyl-L-cysteine, S-nitroso-N-acetyl-DL-penicillamine) and 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO). Such donors may be added to a final concentration of between for example 10nM to 300μM. The half-life of the above mentioned donors vary. The half-life of DEA/NO is, for example, approximately 2 minutes. Donors with shorter half-lives, for example 1 to 5 minutes are preferred and those with half-lives of 2 to3 minutes are most preferred.

Determining whether sGC is stimulated may be carried using any suitable method. Typically sGC activity is determined before and after contacting an axon with a substance capable of stimulating sGC. The activity of sGC can be determined directly. It is generally most convenient to do this by measuring the production of cGMP by sGC. For example, by measuring the conversion of radiolabelled GTP into cGMP. Alternatively or additionally, a pH sensitive probe may be used to determine sGC activity, as H^+ ions are also produced by the enzymatic reaction catalysed by sGC. A further method for measuring the activity of sGC is to use a fluorescent tag on the sGC enzyme. In such a method sGC is modified using recombinant DNA techniques so that the sGC comprises a fluorescent polypeptide domain. The fluorescent properties of the resulting sGC: fluoresent polypeptide enzyme change depending on the activity of the enzyme.

It is most convenient to determine whether sGC is stimulated by measuring cGMP levels before and after contacting an axon with a substance that is capable of stimulating sGC. The production of cGMP may be determined by any suitable technique known to those skilled in the field. For example, radioimmunoassays,

A non-viable axon may be assayed to determine whether any sGC stimulation occurs in that axon. An axon may be rendered non-viable by subjecting it to for example, oxygen deprivation and/or sugar, eg. glucose, deprivation. Typically, it is preferable to use conditions under which irreversible damage to the axon occurs. For example, incubating nerves in a medium with no glucose and gassed with 5% CO_2 in N_2 for 1 hour causes irreversible damage to the majority of axons so incubated (Garthwaite *et al.*, 1999).

Other types of cell known to exhibit sGC stimulation and increase in cGMP formation in response to NO may be used as positive controls. For example vascular enodothelial cells show an increase in cGMP formation on stimulation with NO and could therefore be used as positive control in the assay.

Generally, a viable axon is one which shows greater sGC stimulation than that shown by a non-viable axon. Typically, a viable axon will show an increase in sGC activity of at least 2-fold that shown by a non-viable axon. More preferably, a viable axon will show an increase in sGC activity of at least 25-fold, more preferably 50-fold that shown by a non-viable axon.

Similarly, if modulation of activity of a cGMP target is used to measure sGC stimulation, a viable axon is one which shows greater modulation of activity of a cGMP target than that shown by a non-viable axon.

If cGMP generation is used as a measure of sGC stimulation, a viable axon is generally one which shows a greater increase in cGMP generation than that shown by a non-viable axon. Typically, a viable axon will show an increase in cGMP generation of at least 2-fold that shown by a non-viable axon. More preferably, a viable axon will show an increase in cGMP generation of at least 25-fold, more preferably 50-fold that shown by a non-viable axon.

The magnitude of the sGC stimulation observed may depend on the concentration of sGC stimulator present in the assay. Therefore greater sGC stimulation may be observed when higher concentrations of sGC stimulator are used. A viable axon will preferably show sGC stimulation at low concentrations of sGC stimulator.

The invention also provides a method of identifying a substance capable of

least 500%, at least 1000%, at least 50000%, at least 100000% at a concentration of the protectant of 1μg ml^{-1}, 10μg ml^{-1}, 100μg ml^{-1}, 500μg ml^{-1}, 1mg ml^{-1}, 10mg ml^{-1}, 100mg ml^{-1}. The percentage increase represents the percentage increase in axon viability in a comparison of assays in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage increase in axon viability and concentration of protectant may be used to define a protectant of the invention, with greater increase in axon viability at lower concentrations of protectant being preferred.

Candidate protectants which show activity in assays such as those described above can then be tested in *ex vivo* models and *in vivo* models. A suitable ex vivo model involves dosing an animal with a neuroprotective agent. After a suitable time for absorption and brain penetration of the agent, the animal is killed. The decapitated head is left at normal body temperature for a given interval (eg. 1h) and then the optic nerves are taken out, incubated in vitro and assayed for viability.

Suitable *in vivo* models include traumatic damage to the spinal cord (which damages white matter). Animal models exist for the majority of the indications given below and are well known to those skilled in the art.

Protectants identified by the screening procedures described above may be used to treat any condition associated with white matter damage. Conditions associated with white matter damage include cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, viral infections (eg. human immunodeficiency virus), alcohol abuse, cerebral malaria and motoneurone disease. Additionally, protectants of the invention may be used in the manufacture of a medicament for use in the treatment of one of the above mentioned indications. The condition of a patient suffering from any of the above mentioned conditions can therefore be improved by administration of such a protectant of the invention. A therapeutically effective amount of a protectant of the invention may be given to a human patient in need thereof.

Protectants of the inventon may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges,

a suitable amount of lidocaine hydrochloride.

Solutions for intravenous administration or infusion may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of a protectant is administered to a patient. The dose of a protectant may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the specific protectant, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

The following Example illustrates the invention.

Example

Materials and methods

Optic nerve preparation

Nerves (about 9 mm long) were excised from adult Wistar rats (240-280 g) after decapitation. They were incubated in Erlenmeyer flasks (50 ml capacity) containing 20 ml of an artificial CSF (aCSF) solution composed of (mM): NaCl (120) KCl (2.0), $CaCl_2$ (2.0), $NaHCO_3$ (26), KH_2PO_4 (1.18), $MgSO_4$ (1.19) and glucose (11), continuously gassed with 95% O_2/5% CO_2. The flasks were held in a shaking water bath at 37°C. For the Ca^{2+}-free medium, ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (1 mM) was substituted for $CaCl_2$ and for the Na^+-free medium, 120 mM choline chloride and 26 mM choline bicarbonate replaced NaCl and $NaHCO_3$ respectively.

Oxygen and glucose deprivation.

(4 min). Counterstaining was carried out using Mayer's haemalum for 15 s. The 1 μm thick resin-embedded sections were etched with 1:1 mixture of ethanol and saturated sodium hydroxide in ethanol for 5 min before immunohistochemistry; these sections were counterstained with Mayer's haemalum for 5 min.

Materials

The sheep anti-cGMP antibody was a kind gift from Dr. J. de Vente (Maastricht, Netherlands). Secondary antibodies and the ABC kit were purchased from Vector laboratories (Orton Southgate, Peterborough, UK). DEA/NO was from Alexis Corporation (Bingham, Nottingham, UK) or RBI (through Semat Technical UK Ltd., St. Albans, Herts, UK). Tetrodotoxin was from Latoxan Laboratories (Rosans, France). Lamotrigine, BW619C89 and BW1003C87 were supplied by the Wellcome Research Laboratories (Beckenham, Kent). Other chemicals were from Sigma-Aldrich (Poole, Dorset, UK), BDH/Merck (Poole, Dorset, UK) or Tocris-Cookson (Bristol, UK).

Results

Basal cGMP levels in the rat optic nerves averaged 1.06 ± 0.14 pmol/mg protein (n = 4) and the levels were 3-fold higher in presence of the non-selective phosphodiesterase inhibitor, IBMX (1 mM; 3.55 ± 0.36 pmol/mg protein; n = 8). To test the ability of NO to elevate cGMP levels in this tissue, the NO-donor DEA/NO, which dissociates with a half-life of about 2 min (Morley & Keefer, 1993) was used. Exposure of the nerves for 5 min to DEA/NO (10 nM - 300 μM), in the presence of IBMX, resulted in concentration-dependent accumulation of cGMP to levels that were ultimately more than 50-fold higher than in the unstimulated tissue (Fig. 1a). Half-maximal effects occurred at about 10 μM DEA/NO. The inhibitor of NO-stimulated soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (Garthwaite *et al.*, 1995), at a concentration of 3 μM (10 min preincubation), reduced the cGMP response to 100 μM DEA/NO from 219 ± 23 to 32 ± 1 pmol/mg protein (n = 4) confirming the involvement of this enzyme. In the absence of IBMX, maximal cGMP accumulation (with 100 μM DEA/NO), instead of being more than 200

results were found with Ca^{2+}-free solution (results not shown).

Anoxic damage to optic nerve, assayed using electrophysiology, has been shown to be lessened in the presence of certain antiepileptic drugs (e.g. phenytoin and carbamazepine), local anaesthetics and antiarrhythmic agents (Stys, 1998). The efficacy of these measures is explained by their capacity to block voltage-dependent Na^+ channels. The newer antiepileptic drug, lamotrigine, and the structurally related molecule, BW619C89, block Na^+ channels in a use- and voltage-dependent manner (Xie *et al.*, 1995; Xie & Garthwaite, 1996) and are neuroprotective towards grey matter *in vivo* (Taylor & Meldrum, 1995; Urenjak & Obrenovitch, 1996). Hence, these compounds, and the structurally-related neuroprotectant, BW1003C87 (Meldrum *et al.*, 1992), were tested for their ability to protect the optic nerve against OGD using histology and the NO-stimulated cGMP accumulation.

The compound BW619C89 provided concentration-dependent protection against OGD-induced loss of the cGMP response (Fig. 3), the half-maximal effect being observed at about 6 μM. At the highest concentrations (30-100 μM), the response amplitude was not significantly different from that of control nerves that had not been subjected to OGD. Substantial, though incomplete, protection was also achieved with BW1003C87 (30 μM; 60% protection) and lamotrigine (100 μM; 40% protection) (Fig. 3). On their own, none of the 3 compounds had an adverse effect on the ability of nerves to produce cGMP in response to DEA/NO (Fig. 3 and results not shown). Histology and cGMP immunohistochemistry correlated well with the biochemical results: for example, BW619C89 (30 μM) protected the axons from OGD-induced pathology (Fig. 2h) and loss of axonal cGMP immunostaining (Fig. 2k).

Discussion

The existence of the NO receptor, soluble guanylyl cyclase, in optic nerve was not previously known. Signalling by NO through this mechanism has, however, been described in many other tissues and it appears to be the principal pathway through which physiological NO signalling occurs (Ignarro, 1991; Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997; Hobbs, 1997). The finding that NO

cGMP is synthesised from GTP which exists in equilibrium with adenosine 5'-triphosphate (ATP) intracellularly (Voet & Voet. 1995); consequently, non-viable tissue, lacking high energy phosphates, is unable to generate cGMP in this manner, even if the synthetic enzyme should remain intact. The dependence of the cGMP response on cellular viability has been exploited previously for the identification of the sources and targets of NO in the cerebellum (Garthwaite & Garthwaite, 1987). The significant features of the response in the optic nerve were first, its apparently exclusive location in axons and secondly its magnitude, the two together making NO-induced cGMP accumulation a sensitive marker for optic nerve axon viability. Accordingly, in optic nerves previously subjected to 1 h of OGD, the cGMP response was only 17% of its value in control nerves. The residual cGMP elevation was attributable (on the basis of immunohistochemistry) to the survival and normal behaviour of a subpopulation of axons (seemingly distributed randomly), as opposed to a generalised reduction in the ability of axons to generate cGMP. The extent of functional axonal loss recorded with this technique is in excellent agreement with that recorded electrophysiologically, in which 1 h of OGD caused an 80% loss of the optic nerve compound action potential (Fern *et al.*, 1998). Moreover, the various procedures that were found previously to protect optic nerve axons from OGD to differing extents, as judged by a morphometric method (Garthwaite *et al.*, 1999), all had quantitatively very similar effects on the level of NO-induced cGMP accumulation. The correspondence in the readout of two independent methods (one based on histology, the other on function) lends strong support to their reliability for assessing optic nerve axon pathology.

Interpretation of the findings with respect to the mechanism of OGD-induced damage follows that proposed from very similar findings made previously using the quantitative morphometric method (Garthwaite *et al.*, 1999). In brief, the findings indicate that the damage is dependent on the activity of voltage-dependent Na^+ channels and an influx of Ca^{2+} into the axoplasm and are consistent with a mechanism proposed to account for anoxia-induced damage, namely influx of Na^+ followed by reversal of the Na^+-Ca^{2+}-exchanger leading to a Ca^{2+} overload of the axoplasm (Stys, 1998). The lesser protective efficacy of Na^+-free aCSF may be

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(ii) determining the viability of the axon by a method according to any one of the preceding claims; and

(iii) determining thereby whether the test substance can protect the axon from loss of viability.

12. A substance identified by a method according to claim 11.

13. A substance according to claim 12 for use in a method of treatment of the human or animal body by therapy.

14. A substance according to claim 13 for use in a method of treatment of a condition associated with white matter damage.

15. A substance according to claim 14 for use in a method of treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.

16. Use of a substance according to claim 11 in the manufacture of a medicament for use in the treatment of a condition associated with white matter damage.

17. Use of a substance according to claim 11 in the manufacture of a medicament for use in the treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.

18. A method of treating a host suffering from a condition associated with white matter damage, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 11.

19. A method of treating a host suffering from cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 11.

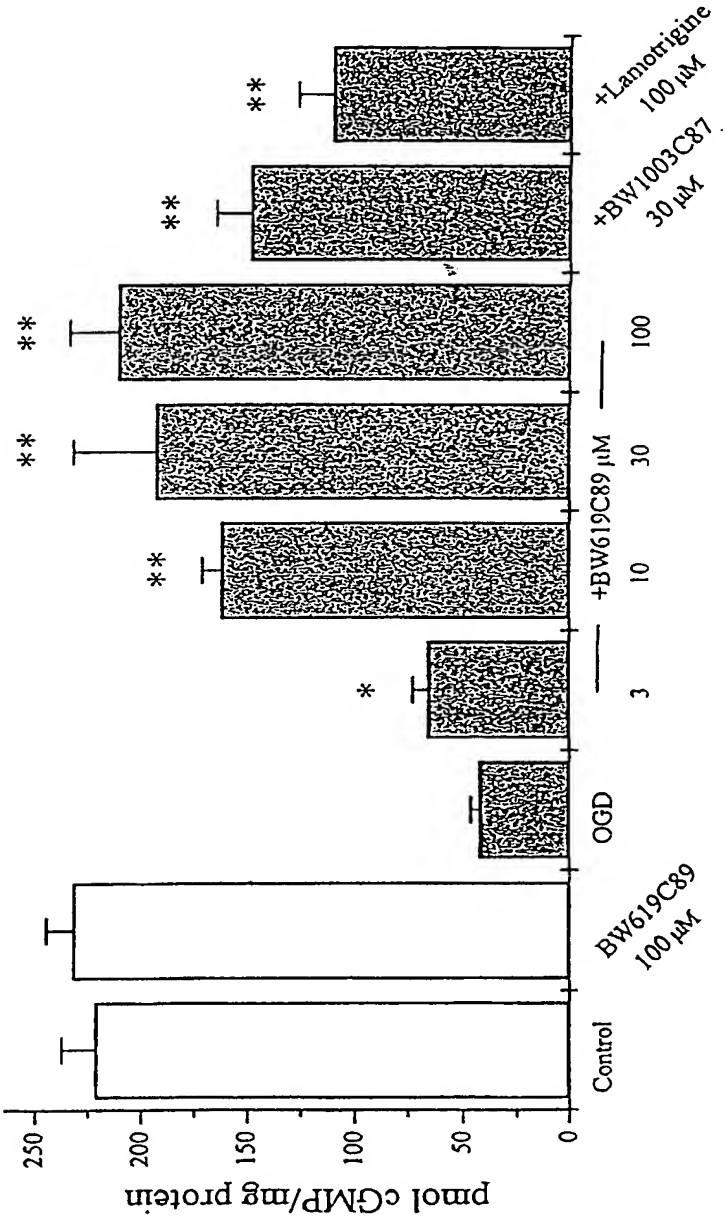

Figure 2

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	XIE X M ET AL: "State-dependent inhibition of Na+ currents by the neuroprotective agent 619C89 in rat hippocampal neurons and in a mammalian cell line expressing rat brain type IIA Na+ channels." NEUROSCIENCE, vol. 73, no. 4, 1996, pages 951-962, XP000993069 ISSN: 0306-4522 cited in the application abstract	12-19
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P,X	GARTHWAITE GITI ET AL: "Nitric oxide stimulates cGMP formation in rat optic nerve axons, providing a specific marker of axon viability." EUROPEAN JOURNAL OF NEUROSCIENCE, vol. 11, no. 12, December 1999 (1999-12), pages 4367-4372, XP000990808 ISSN: 0953-816X the whole document	1-19
P,X	GARTHWAITE G ET AL: "Monitoring rat optic nerve axon viability using nitric oxide-stimulated cGMP accumulation: Application to the mechanism of ischaemic damage." SOCIETY FOR NEUROSCIENCE ABSTRACTS., vol. 25, no. 1-2, 1999, page 1841 XP000990791 29th Annual Meeting of the Society for Neuroscience.;Miami Beach, Florida, USA; October 23-28, 1999 ISSN: 0190-5295 the whole document	1-19

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 12-19

Claims 12-19 relate to substances defined by reference to a desirable characteristic or property, namely being identified by the method of claim 11, i.e. contacting an axon with a test substance under conditions that in the absence of the test substance would lead to a decrease in viability, determining the viability of the axon by a method according to claims1-10, and determining thereby whether the test substance can protect the axon from loss of viability. Claims 12-19 relate furthermore to medical uses and methods of treatment related to these substances. Claims 12-19 cover all substances having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no such substances.
Furthermore, even known substances such as lamotrigine (Xie et al. (1995), Pfleugers Arch. Eur. J. Physiol. 430, 437-446), compound 619C89 (Xie et al. (1996), Neuroscience 73, 951-962) and BW 1003C87 (Meldrum et al. (1992), Brain Research 593, 1-6) fall under the scope of the claims. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the subject-matter for which protection is sought in claims 12-19 is impossible.
Consequently, no complete search has been performed on these claims.

It is also pointed out that claims 18-19 relate to treatment of human or animal body by therapy (Rule 39.1(iv) PCT).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.